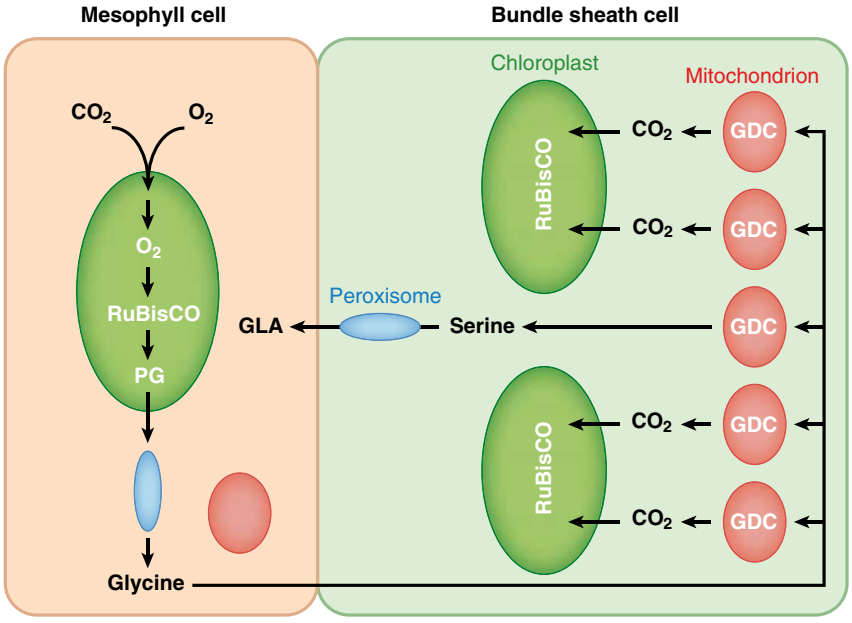

Figure 5. C_2 photosynthesis.
Oxygen binds to Rubisco in chloroplasts of the mesophyll to produce PG. This is then transported to the peroxisome where it is converted to glycine. Glycine is then transported to the centripetally arranged mitochondria of the bundle sheath, where two molecules of glycine are converted to one of serine plus one CO_2. The serine is imported in to the peroxisome where it is converted to GLA. The CO_2 is recaptured by centrifugally arranged chloroplasts. PG, phosphoglycolate; GDC, glycine decarboxylase; GLA, glycerate; green ovals, chloroplasts; pink ovals, mitochondria. (Figure used with permission from R.F. Sage.)

regulation of Rubisco and PEP carboxylase.

Conclusions

Despite its economic and ecological importance, and despite years of research on all aspects of the C_4 pathway, one major obstacle lies in the way of using it to increase crop yield — we do not know how it is controlled. This limits our ability to understand how the pathway evolved and how it can be manipulated in crops. If we are to use C_4 photosynthesis to improve crop yields, a deeper understanding of the controls of the pathway will be required. This understanding is likely to come from a combination of genetic, genomic, and comparative evolutionary studies. The controls of leaf anatomy and histology remain poorly understood. In particular, the regulation of vein density, size of the bundle sheath, and organelle number are unknown, although clearly they are related to the dynamics of auxin, and may also be affected by duration of the activity of regulatory genes. In addition, the photorespiratory cycle may provide some clues. If localization of the C_2 cycle to bundle sheath cells establishes a pre-pattern for C_4, then the mechanisms by which those enzymes are localized are important aspects of the pathway, as is the evolutionary history of their regulation.

Further reading

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Artificial microRNAs reveal cell-specific differences in small RNA activity in pollen

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Pollen formation, while critical for the success of plant reproduction, also represents an important paradigm for differential cellular development within small groups of cells. In *Arabidopsis thaliana* pollen, the male meiotic product first divides asymmetrically to form a vegetative and a generative (germ) cell, the latter then dividing to generate two sperm cells. Here we have used artificial microRNAs to study small RNA processing in the different pollen cell types. Our data suggest that translational repression by small RNAs is enhanced in the sperm. This work also provides insights into germline RNA movement and the cell-autonomous action of microRNAs.

Pollen development is accompanied by changes in the expression of many non-coding small RNA biogenesis genes [1,2] and in target cleavage by diverse microRNAs [1]. To determine whether the vegetative cell and the germline within the pollen grain differ in microRNA processing and target silencing, we generated artificial microRNA (amiR) constructs driven by cell type-specific promoters. AmiRs are effective tools for gene silencing in plants; by exploiting a non-coding RNA backbone that forms a precursor 'hairpin', the region excised by DICER-LIKE1 to form a mature microRNA can be engineered to specifically target RNAs through ARGONAUTE-mediated interactions [3]. Thus, specific RNAs can be 'knocked-down', generally by cleavage, the predominant mode of microRNA action in plants.

First, to test our experimental system we designed an artificial microRNA construct (amiRGFP) to silence a GFP marker expressed specifically either in the germline (sperm cells) or in the accessory vegetative cell of pollen. To express the amiRGFP we chose the tomato LAT52 promoter,

as it is effective in *Arabidopsis* and, when driving the GUS reporter, it is first expressed in late microspore development and subsequently accumulates to a higher level in the vegetative cell [4]. The expression of our PromLAT52:H2B-GFP construct in *Arabidopsis* followed this pattern, but also revealed that GFP was inherited from the microspore nucleus into the generative cell — the plant male germline (Supplemental Figure S1A–E).

Importantly, in this work we also utilised a novel promoter that is specific to the vegetative cell. Analysis of expression data of At2g24370, encoding a putative serine/threonine protein kinase, revealed that it is expressed late in male gametophyte development, but not in sperm cells [2]. Analysis of GFP expression under its promoter established that expression was restricted to the later stages of vegetative cell development (Supplemental Figure S1F–J), hence we named this PromVCK1 (vegetative cell kinase1). We also used the promoter of the *MGH3* gene, a histone H3 variant gene with well-characterised generative and sperm cell-specific expression at high levels [5]. The ability of these pollen-specific promoters to permit generation of mature artificial microRNAs is shown in Supplemental Figure S1K.

Knockdown of vegetative cell-specific GFP expression under PromVCK1 control, by the amiRGFP under the LAT52 promoter, revealed efficient reduction of mRNA and protein levels (Table 1). By contrast, knockdown of GFP under PromMGH3 control, by amiRGFP under the MGH3 promoter, led to a significant reduction in GFP protein levels, but without a concomitant reduction in mRNA levels. This result indicates that in the sperm the amiRGFP is working primarily by translational repression, as opposed to the canonical cleavage-induced mRNA turnover in the vegetative cell.

To investigate further the translational control in sperm cells we analysed transcriptome data for *SUO*, which encodes a GW-repeat protein involved in promoting translational repression by microRNAs [6] and found both *SUO* and its close paralogue (At3g48060) to be at their highest levels in sperm when compared with the vegetative cell or the sporophytic phase of plant development [2] (Supplemental Table S1). Translational repression is sensitive to *SUO* dosage [6] and thus *SUO* upregulation constitutes a second strand of evidence supporting the existence of active translational repression in *Arabidopsis* sperm. A further component recently shown to be important for translational repression of plant microRNAs on the rough endoplasmic reticulum [7], *ALTERED MERISTEM PROGRAM1* (*AMP1*), encodes a homologue of human glutamate carboxypeptidase II, which is also enriched in sperm cells compared with the vegetative cell of mature pollen (Supplemental Table S1). Translational repression is known to be important in plant reproduction as paternally derived *SHORT SUSPENSOR* transcripts, essential for regulation of the first asymmetric division of the zygote, are translationally repressed in sperm and only translated upon fertilisation [8].

Previous experiments using artificial microRNAs targeting pollen gene expression led to the development of a model for small RNA function in the male gametophyte [9]. In this work, Slotkin *et al*. used PromLAT52 to direct amiR expression combined with sperm-specific GFP expression directed by PromGEX2. This resulted in knockdown of GFP in sperm, and given that PromLAT52 is highly expressed in the vegetative cell, it was proposed that knockdown arises through small RNA transfer from the vegetative cell to the germline. These data were used to support the notion that transposable element (TE)-derived siRNAs, produced by epigenomic changes in the vegetative cell, move to the germline where they reinforce TE silencing by RNA-dependent DNA methylation [9]. Our experiments using PromLAT52 resulted in silencing of germline GFP (as in Slotkin *et al.* [9]) but, against expectation, expression of the amiRGFP using PromVCK1 failed to silence germline-specific GFP expression directed by PromMGH3 (Table 1).

Although we cannot rule out that continuity may exist transiently between the vegetative cell and generative cell prior to PromVCK1 activation, an equally reasonable explanation for germline silencing directed by PromLAT52 is that amiRNAs, derived from early PromLAT52 expression in the microspore [4] (Supplemental Figure S1A–E), are inherited by the germline. Furthermore, the observation that sperm cells display a microRNA profile

Table 1. Analysis of GFP protein and transcript levels in Prom:H2B-GFP target reporter lines harbouring Prom:amiR effector constructs (see also Figure S1).

				amiR effector in target reporter line			Target reporter line alone				
amiR effector	Target reporter	[1]T1 (n)	[2]%KD	[3]ID	[4]GFP protein ±SE	[5]GFP transcript ±SE	[6]ID	[4]GFP protein ±SE	[5]GFP transcript ±SE	[7]amiR KD efficiency GFP protein (%)	[8]amiR KD efficiency GFP transcript (%)
ProLAT52 :amiRGFP	ProVCK :H2B-GFP	36	78	A2 B6	0.2±0.0 0.2±0.0	3.4±0.1 3.1±0.0	A6 B3	25.4±1.6 38.0±1.2	21.2±0.0 24.5±0.2	99.4	85.8
ProLAT52 :amiRGFP	ProMGH3 :H2B-GFP	26	73	C1 C6	0.2±0.1 0.2±0.0	2.8±0.1 3.5±0.0	B4 B6	2.1±0.1 2.1±0.1	4.1±0.0 3.7±0.0	90.8	19.2
ProMGH3 :amiRGFP	ProMGH3 :H2B-GFP	41	39	B5 D2	0.5±0.0 0.9±0.1	5.0±0.1 4.5±0.2	A1 D5	3.3±0.2 3.2±0.2	3.9±0.1 7.4±0.2	79.6	15.9[NS]
ProVCK :amiRGFP	ProMGH3 :H2B-GFP	20	0	B4 B5	1.9±0.1 2.2±0.1	4.4±0.1 3.8±0.1	A1 D3	2.1±0.1 2.3±0.1	4.7±0.1 3.3±0.1	9.8[NS]	-2.5[NS]

[1]Number of T1 lines analysed. [2]Proportion of T1 lines with reduced GFP signal. [3]ID of lines homozygous for amiRGFP and target reporter constructs. [4]Mean GFP fluorescence in pollen nuclei. [5]Mean GFP transcript level by q-RT-PCR. [6]ID of lines homozygous for target reporter constructs. [7]Mean GFP signal in amiR effector line/mean GFP signal in segregating target reporter line without amiR effector construct X 100. [8]Mean GFP transcript level in amiR effector line/mean GFP transcript level in segregating target reporter line. [NS]Not significantly different from control target reporter line ($P < 0.05$).

distinct from whole mature pollen [11] is consistent with our finding that miRNAs do not appear to act across the vegetative/sperm cell interface.

Our data clearly reveal the presence of different and cell-autonomous differences in miRNA activity in the mature pollen grain of *Arabidopsis*. They further highlight the importance of promoter specificity when using amiRs to target mRNAs in rapidly dividing and differentiating systems, and the caution required when interpreting cell-to-cell transfer data from amiR experiments.

Supplemental Information

Supplemental Information includes experimental procedures, one figure, one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.05.055.

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Cohesin cleavage is insufficient for centriole disengagement in *Drosophila*

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Centriole disengagement is thought to act as a licensing mechanism restricting centrosome duplication to once per cell cycle [1] and to depend on cleavage of the cohesin complex by separase [1–3]. Whether this is a conserved mechanism in eukaryotic cells remains to be determined. We show that artificial cohesin cleavage in *Drosophila* embryos fails to cause detectable centriole disengagement. In contrast, inhibition of Cyclin-dependent kinase (Cdk1) triggers rapid disengagement in metaphase-arrested embryos. Our results raise the possibility that in these early embryonic divisions centriole engagement depends on Cdk1 activity, not cohesin.

Mother and daughter centrioles are kept tightly together and in an orthogonal arrangement (engaged) from the time of their duplication until the subsequent mitosis, and their disengagement occurs during later stages of mitosis. The molecular mechanisms behind centriole engagement during S-phase and their disengagement during mitotic exit are far from being understood. But it has been proposed that during mitotic exit, centriole disengagement is mediated by separase [1,2], a thiol protease known to promote disjunction of sister chromatids at anaphase onset [4].

Until recently, the only known target of separase was the cohesin complex, a ring-shaped multisubunit protein complex (composed of Smc1, Smc3, Rad21/Scc1 and Scc3/SA) known to entrap sister DNA molecules inside its ring and thereby promote sister chromatid cohesion until anaphase onset [4]. Separase-mediated cleavage of cohesin's kleisin subunit (Scc1/Rad21 in mitotic cells) leads to opening of the cohesin ring and subsequent sister chromatid disjunction. The fact that separase has been implicated in the process of centriole disengagement [1,2] led to the speculation that the cohesin complex could be the molecular 'glue' that holds mother/daughter centrioles together from the time of their duplication until mitotic exit, in a way similar to how these complexes hold sister DNA molecules together. Indeed, many reports have suggested that cohesin interacts with some centrosomal proteins, that cohesin and other cohesion proteins localize to the centrosome, and that cohesin depletion leads to centrosomal defects ([5] and references therein). Nevertheless, attempts to clearly define the role of cohesin in this process have led to conflicting results. While initial studies report that expression of a non-cleavable cohesin complex (NC-Rad21) in HeLa cells does not prevent disengagement, suggesting that this process depends on a yet undefined separase target other than Rad21 [2], recent studies using purified centrioles from mammalian cells suggest that centriole engagement is dependent on cohesin's integrity [3].

We have recently been able to artificially reproduce a bona fide mitotic exit from metaphase-arrested embryos [6]. In this experimental setup, separation of sister chromatids is achieved using a system to inactivate cohesin complexes by an exogenous protease (Tobacco Etch Virus, TEV) [6,7]. Proper mitotic exit, in turn, is driven by artificial downregulation of Cdk using high doses of the cyclin-dependent kinase inhibitor p27. Cohesin cleavage and inhibition of Cdk are both necessary and sufficient to reproduce a bona fide anaphase and mitotic exit with normal kinetics of chromatid separation, proper relocation of the Chromosome Passenger Complex to the spindle mid-zone, normal inactivation of the Spindle Assembly Checkpoint and timely chromosome decondensation and nuclear envelope reformation [6].

To evaluate whether this artificially induced mitotic exit is also accompanied by proper centriole disengagement, we have repeated the same experiments in embryos previously injected with mRNA coding for a fluorescent centriole marker (Sas4-EGFP). In most somatic cell types, disengaged centrioles are known to remain tightly joined by cohesion fibres during interphase, preventing centriole separation.